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THIOL PEROXYL RADICAL FORMATION FROM THE REACTION OF CYSTEINE THIYL RADICAL WITH MOLECULAR OXYGEN: AN ESR INVESTIGATION

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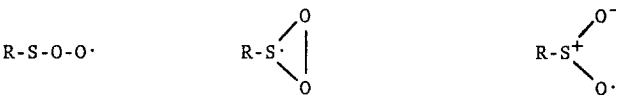
SUMMARY. Using Electron Spin Resonance (ESR) spectroscopy, we have identified the cysteine thiol peroxyl radical (CysSOO·) at low temperatures in two aqueous glasses. This radical shows a typical peroxyl radical ESR spectrum, but unlike carbon-based peroxyl radicals has a violet color (λ_{max} = 540 nm) and forms a new radical showing a singlet ESR spectrum when photobleached with visible light. The cysteine peroxyl radical reacts to form the cysteine sulfinyl radical (CysSO·) in the glass which allows warming to 165K. ^{17}O isotopic substitution studies indicate dissolved molecular oxygen is the source of oxygen in CysSOO·. Anisotropic g-values and the parallel anisotropic ^{17}O hyperfine couplings for this radical are reported.

The thiol peroxyl radical (I) has been proposed as an important transient species in glutathione radical chemistry.[1] Pulse radiolysis studies indicate that thiyl radicals (RS·) from glutathione and other sulfhydryls react with molecular oxygen to form a product that possesses a visible absorption at ca. 550 nm.[1-3] Several workers have suggested the product is the sulfur based peroxyl radical (I), while von Sonntag has pointed out that such a long wavelength aborption is unkown for other peroxyl radicals and has proposed two other possible structures (II,III) for this product.



I II III

Using Electron Spin Resonance spectroscopy and frozen aqueous solutions of relatively high concentrations of thiols we observed in previous work that thiyl radicals react with molecular oxygen to form sulfinyl radicals (RSO·).[4,5] The expected thiol peroxyl radical was not detected although we suggested it as a likely intermediate or transition state in the formation of a sulfinyl radical.

We report here the ESR detection and characterization in frozen aqueous glasses of the cysteine thiyl peroxyl radical (CysSOO·) formed by the reaction

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of cysteine thiyl radical (CysS·) with dissolved molecular oxygen, and which is observed only when the concentration of thiol in the sample is relatively low. CysSOO· shows a visible absorption at 540 nm and has an ESR spectrum is observed only when the concentration of thiol in the sample is relatively low. CysSOO· shows a visible absorption at 540 nm and has an ESR spectrum characteristic of a peroxyl radical. This lends support to I as the type of species initially formed by the reaction of thiyl radicals with oxygen.

MATERIALS AND METHODS

L-cysteine HCl monohydrate obtained from Sigma and labeled gaseous oxygen (37 atom % ^{17}O) obtained from Icon were used without further purification. Solutions of cysteine HCl monohydrate (0.6 mM to 60 mM) in 12 M LiCl (D_2O) or 8 M $NaClO_4$ (D_2O) were rapidly frozen in liquid N_2 to form glasses.[6,7] $K_3[Fe(CN)_6]$ was employed in LiCl solutions to scavenge electrons; all samples in $NaClO_4$ were photobleached at 77K to remove electrons before ESR spectra were recorded. Samples were also bubbled with N_2 or O_2 for 1-2 minutes as required. For experiments with ^{17}O enriched O_2, samples were first thoroughly degassed and then sealed under approximately 0.7 atm pressure O_2. After γ-irradiation at 77K for doses of ca. 0.2 Mrad, samples were annealed to various temperatures directly in the ESR cavity using a variable temperature accessory. A Varian Century ESR spectrometer with an E-4531 dual cavity was employed. Unless otherwise noted, spectra were recorded ca. 2 mw microwave power. Hyperfine values and g-values were measured vs. Fremy's Salt with A(N)=13.09 G and g=2.0056.

RESULTS

Figure 1 shows the ESR spectra from frozen γ-irradiated aqueous (D_2O) solutions of L-cysteine (6 mM) in 12 M LiCl containing $K_3Fe(CN)_6$ (5 mM). The initial spectrum at 130K found in both oxygenated and deoxygenated samples is almost entirely due to $Cl_2^{\cdot -}$ radical. (Figure 1A) Annealing deoxygenated samples to 145K results in the appearance of a highly anisotropic spectrum (g_z = 2.13, g_x = 2.01, g_y = 2.00) due largely to the cysteine thiyl radical, which is formed by the one electron oxidation of cysteine by $Cl_2^{\cdot -}$ (Figure 1B).

$$CysSH + Cl_2^{\cdot -} \longrightarrow CysS\cdot + H^+ + 2Cl^- \qquad (1)$$

The value found here for the characteristically broad g_z feature (g_z= 2.13) is the same as that reported for cysteamine thiyl radical and mercaptoacetic acid thiyl radical in aqueous matrices.[8] The broad feature near the free electron g-value is also characteristic of randomly oriented thiyl radicals.[4,5] For an oxygen saturated sample CysS· develops at 147K and upon further annealing to 155K reacts to form a new species whose spectrum is shown in Figure 1C. This spectrum originates from a radical with an anisotropic g-tensor, with principal values (at 140K) g_x=2.0027, g_y=2.0090, g_z=2.035. and resolution of the line components suggests there are no significant hyperfine couplings present. This spectrum is associated with a violet color in the

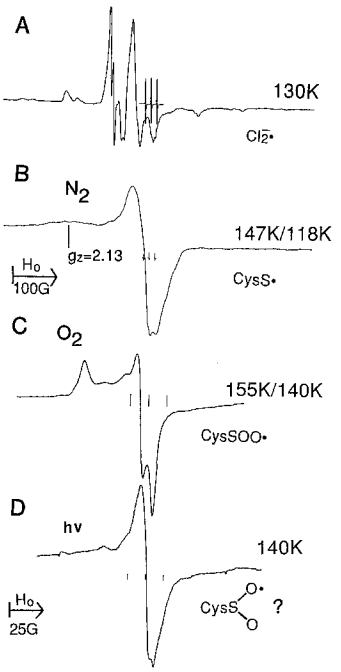

FIGURE 1. The ESR spectra of γ-irradiated (77K) L-cysteine (6 mM) in 12 M LiCl in D_2O with $K_3Fe(CN)_6$ (5 mM). (A) Cl_2^{-}· found initially in all samples (after photobleaching to remove trapped electrons). (B) CysS· formed by annealing a deoxygenated sample. Microwave power = 80 mw. (C) CysSOO· formed by annealing an oxygen saturated sample. (D) Photobleaching the sample in (C) results in the singlet shown in (D), which is tentatively assigned to $CysSO_2$·. Where two temperatures are shown, they are 'highest annealing temperature/spectrum recording temperature'. The three small field calibration lines are 13.09G apart with the central line at g=2.0056. Note that the scale of (A) and (B) is different from that of (C) and (D).

sample (visible in the absence of the colored $K_3Fe(CN)_6$) which is easily photobleached with visible light. We assign the spectrum shown in Figure 1C to the cysteine thiol peroxyl radical, $(NH_3^+)(CO_2^-)CHCH_2SOO$· (CysSOO·) formed by the reaction of molecular oxygen and cysteine thiyl radical, reaction 2.

$$CysS\cdot + O_2 \longrightarrow CysSOO\cdot \qquad (2)$$

Figure 1D shows the spectrum that results when a sample giving spectrum 1C is photobleached with an incandescent lamp. The color is lost and a new spectrum consisting of a singlet at 2.0053 is obtained. The cysteine sulfonyl radical, $CysSO_2$·, (III), a likely rearrangement product, would be expected to give a spectrum at this g-value and show no resolved hyperfine coupling, as found.[9] Since, there are other possible structures which could explain this singlet (for example, II), such an identification must be considered tentative. However, it is clear that visible light causes the rearrangement of the thiol peroxyl radical to a new species.

Figures 2A-B show the spectra obtained when a γ-irradiated oxygenated sample of L-cysteine (6 mM) in 8 M $NaClO_4$ is annealed. The initial spectrum obtained at 108K (not shown) is due largely to $O^{\cdot -}$, with minor line components present from the 8 M $NaClO_4$ medium. Annealing to 160 K results in formation of the cysteine thiyl peroxyl radical (Figure 2A). Samples showing this spectrum also have an intense violet color. A visible spectrum taken of this species gives a broad absorption with λ_{max} = 540 nm (Figure 3). When the sample is warmed to 165K and left at this temperature for a few minutes, the spectrum from the cysteine sulfinyl radical, CysSO·, develops (Figure 2B1)[4,5] with loss of the 540 nm absorption. Varying the concentration of cysteine (0.6 mM to 60 mM) and annealing samples results in the rapid and substantial conversion of CysSOO· to CysSO· at 60 mM cysteine and a slow and only partial conversion at 0.6 mM cysteine. This conversion was much less in the 12 M LiCl glass, perhaps due to the fact that this glass softens with radical loss at a lower temperature. Photobleaching samples giving an ESR spectrum identical to that in 2A gives rise to the ESR spectrum shown in Figure 2B2, which, as mentioned above, is tentatively identified as the cysteine sulfonyl radical. Photobleaching also results in the loss of the visible absorption at 540 nm (see Figure 3B).

Figure 4 shows the spectrum obtained when a sample of L-cysteine in 8 M $NaClO_4$ saturated with isotopically enriched O_2 (37 atom % ^{17}O) is γ-irradiated and annealed to 160K. At this temperature the sample is violet and the centrally located ^{16}O ESR spectrum corrsponds to that shown in Figure 2A, thus the predominant radical present is CysSOO·. Two sets of ^{17}O couplings are

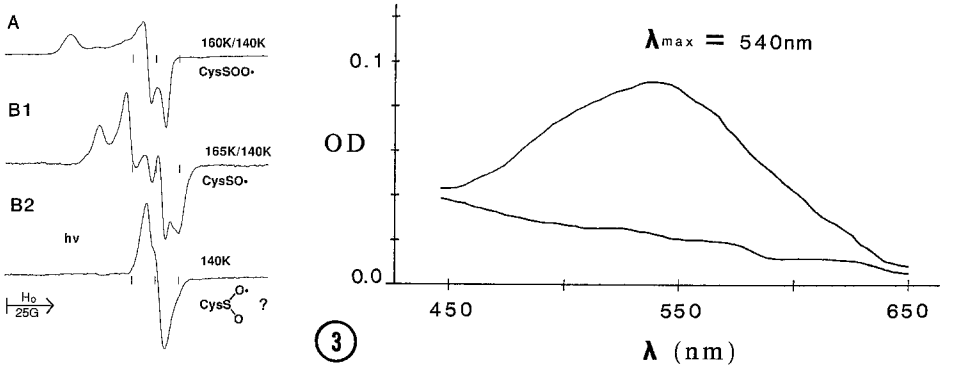

Figure 2. The ESR spectra of γ-irradiated (77K) L-cysteine (6 mM) in 8 M $NaClO_4$ in D_2O. (A) CysSOO· obtained by annealing to 160K. (B1) Further annealing results in conversion to CysSO·. (B2) Photobleaching samples showing the spectrum in (A) results in the singlet shown which is tentatively assigned to $CysSO_2$·. Computer subtraction was used to eliminate some signal intensity from CysSO· from spectra (A) and (B2). No additional CysSO· formed on photobleaching.

FIGURE 3. The visible spectrum of CysSOO· in 8M $NaClO_4$ at 77K (upper trace). The spectrum immediately after exposure of the sample to one minute of light from an incandescent lamp (lower trace).

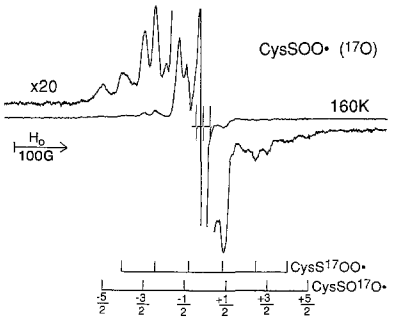

FIGURE 4. The ESR spectrum of CysSOO· obtained from a γ-irradiated frozen solution of cysteine (6 mM) in 8 M $NaClO_4$ saturated with isotopically enriched (37 atom % ^{17}O) O_2 and annealed to 160 K. The spectrum is a superposition of the spectra of CysSOO· (40%), $CysS^{17}OO\cdot$ (23%), $CysSO^{17}O\cdot$ (23%), and $CysS^{17}O^{17}O\cdot$ (14%). The components due to the double substituted species are too weak to be observed even under x20 expansion. The six hyperfine line components expected from each of the two different ^{17}O atoms (spin 5/2) are evident.

evident in the spectrum, as indicated by the stick diagrams in the figure. These correspond to the two monosubstituted forms of the radical, $CysS^{17}OO\cdot$ and $CysSO^{17}O\cdot$. The measured ^{17}O parallel anisotropic hyperfine couplings, centered around g_x=2.003, are $A_x(^{17}O_I)$=78G and $A_x(^{17}O_{II})$=62G at 160K. At 77K, where motional averaging of the hyperfine coupling is diminished, $A_x(^{17}O_I)$ = 81G and $A_x(^{17}O_{II})$ = 64G. In carbon based peroxyl radicals the outer oxygen has the larger ^{17}O coupling[10-12] and ab initio calculations[13] suggest the same is true for thiyl peroxyl radicals. The A_y and A_z ^{17}O couplings are masked by the ^{16}O spectrum; based on the linewidths of the ^{16}O spectrum, we estimate the maximum value for these couplings to be approximately 4 G.

DISCUSSION

Our identification of the sulfur based peroxyl radical in cysteine, CysSOO·, is based on the following points. First, the direct percursor to this species is the cysteine thiyl radical (CysS·) and CysSOO· forms only when oxygenated samples containing the thiyl radicals are annealed until dissolved oxygen becomes mobile. Second CysSOO· shows a ESR spectrum with g-values typical for a peroxyl radical and a visible absorption at 540 nm which lies in the range reported from pulse radiolysis studies for other thiol peroxyl radicals.[1-3] Finally, in a 8M $NaClO_4$ glass, CysSOO· converts to the sulfinyl radical, CysSO·, on annealing to temperatures at which radical migration becomes possible. Reactions 2, 3A and 3B are consistent with our results.

$$\text{CysSOO}\cdot \xrightarrow{h\nu} \text{CysSO}_2\cdot \qquad (3A)$$

$$\text{CysSOO}\cdot + \text{CysSH} \longrightarrow \text{CysSO}\cdot + \text{CysSOH} \qquad (3B)$$

In our previous work at high thiol concentrations relative to those used here, we observed sulfinyl radicals and did not detect thiyl peroxyl radicals. This

observation is consistent with the bimolecular reaction (3B) proposed for formation of CysSO· from CysSOO·.

In spite of substantial spectral similarity between CysSOO· and typical carbon based peroxyl radicals, ROO·, our experiments distinguish between them. Our samples are prepared under conditions in which the formation of carbon centered radicals (R·) is suppressed, hence formation of a substantial amount of carbon based peroxyl radicals is unlikely. CysSOO· is violet, unlike any ROO· radical and undergoes a rearrangement when exposed to visible light, a phenomenon which is unknown for carbon based peroxyl radicals. Due to the lack of significant hyperfine couplings, the ESR spectra of CysSOO· show slightly better resolution of the g-factor anisotropy than that typically found for carbon based peroxyl radicals. Further, the difference in the ^{17}O parallel anisotropic hyperfine couplings (81G - 64G = 17G at 77K) found here for CysSOO· is smaller than those we have found for carbon based peroxyl radicals (30G or more).[12] Preliminary studies of glutathione, cysteamine and penicillamine in the same systems as employed in this work show the production of a colored thiol peroxyl radical from the corresponding thiyl radicals and oxygen. Further investigations of the thiol peroxyl intermediate are in progress.

ACKNOWLEDGMENT

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